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STUDIES ON THE CHEMISTRY OF ANAPHYLAXIS.*

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UP to the present time much of the work done upon the hypersensitization or anaphylaxis reaction has, of necessity, been of a pioneer nature, and directed with the object of establishing the main facts of the subject in a satisfactory manner, in order that these may serve as a foundation for more speculative investigations into the principles underlying the phenomena observed. The reaction having first been discovered in connection with the experiments on and use of antitoxic serum, and the most obviously important feature of the reaction being its bearing on the clinical use of sera, most of the studies so far recorded have concerned investigations with horse serum, although enough other substances have been tried to indicate that the property of sensitization and intoxication is common to many if not to all proteins. As yet, however, but little has been done in the direction of investigation of the exact chemistry of the substances involved in the reaction and of the chemistry of the reaction itself. The complex nature of the solutions commonly used in anaphylaxis experiments, and the wonderfully minute quantities of substance necessary to produce the phenomena, have both discouraged investigation upon the chemical side of the process. In the following article will be recorded statements that have been made by other investigators which touch upon the chemistry of anaphylaxis, and also certain of the results so far obtained in a series of experiments that have been planned and are being carried on in an attempt to systematically investigate this subject.

The pioneer experiments of Richet (1902), to whom we owe the name anaphylaxis, indicated that the reaction concerned proteins. He observed the phenomenon of hypersensitization in connection with his studies of extracts of *actinia*, and found that only the protein-containing part of the extract exhibited this phenomenon.

* Received for publication September 8, 1908.

More recent studies by the same investigator¹ demonstrated the impossibility of producing anything resembling anaphylaxis with non-protein substances, such as cocaine and apomorphine. Rosenau and Anderson, in their first paper² recorded that the toxic principle of horse serum withstands heating at 60° for six hours, but that it is destroyed by heating to 100° for 15 minutes; that it will pass through a Pasteur-Chamberland filter, that drying does not destroy it, and that it remains in the globulin precipitate that contains the antitoxin as prepared by Gibson's method, and that X-ray exposure ("40 amperes, 4 inches from the tube for 40 minutes") had no evident effect. They support the view that the substance which sensitizes and the substance which poisons are one and the same, and in a later communication³ stated that they had obtained no results requiring any alteration in this "working hypothesis." They found that the filtrate from horse serum "after precipitation with ammonium sulphate" (concentration not stated) still possesses sensitizing powers; formaldehyde does not appear to modify the sensitizing property, and the sensitizing property did not seem to dialyze through a collodion sac placed in the abdominal cavity of a guinea-pig. They also make the following statement: "We added a number of different ferments, alkaloids, and simpler chemical substances to horse serum in order to modify, destroy or neutralize its toxic action. All these attempts proved unavailing. The following ferments were used: Taka-diastase, pancreatin, rennin, myrosin, invertin, emulsin, pepsin in acid solution, pepsin in alkaline solution, ingluvin, malt and papain. The ferments were added to the horse serum, and allowed to stand at 15° C. over night." In view of the strong inhibitive action that unheated serum exerts upon the proteolytic enzymes, and the slow rate of action of enzymes at 15° C., these negative results with proteolytic enzymes do not imply that the active agent is not of protein nature. Freezing at 15° F. did not modify the toxic substance. They also found that "hemoglobin, egg albumin, milk and extract of peas are quite as active as horse serum. Peptone seems to have slight sensitizing and poisonous properties; leucine and tyrosine none at all. The reaction following the second injections of proteid matter in the guinea-pig appears then to be common to

¹ Richet, *Presse médicale*, 1908, No. 24, p. 185.

² *Hygienic Laboratory Bulletin*, No. 29, April, 1906.

³ *Jour. Med. Res.*, 1907, 16, p. 381.

the higher forms of albuminous substances no matter from what source." Extracts from bacterial masses and yeast cells were found to produce the typical sensitizing and intoxicating effects.

These early experiments indicate strongly that the anaphylaxis reaction, in common with the various reactions of immunity, concerns protein substances, and, like the precipitin reaction, is exhibited by all natural proteins. That the anaphylaxis reaction is essentially dissimilar to the precipitin reaction, is, however, shown by the fact that the blood of either sensitized or intoxicated animals does not exhibit the phenomenon of fixation of complement (Gay and Southard),¹ and Lewis² states that the refractory stage following intoxication "depends upon a combination of reactions which is without well-known analogy." Whatever the relation of anaphylaxis to the various phenomena of immunity may be, however, there seems to be at least this in common—all concern proteins.

Gay and Adler³ have made attempts to isolate from horse serum the protein or proteins concerned in the reaction, with the following results: On adding ammonium sulphate solution to horse serum to the extent of one-third saturation the precipitate obtained (euglobulin) is actively sensitizing but is not toxic. The proteins precipitated in subsequent fractions as the ammonium sulphate is added until saturation, are found to have less and less power to sensitize but are progressively more toxic. The last fraction, obtained on complete saturation, is at least as highly toxic as whole serum and distinctly less sensitizing. These results are believed by Gay and Adler to indicate that the sensitizing and toxic substances in serum are quite distinct from one another, and although they have been unable to obtain a protein that was toxic but not sensitizing this failure is ascribed to the very minute dose of serum that is necessary to produce sensitization. The sensitizing euglobulin which they obtained upon one-third saturation of the serum is looked upon as a pure sensitizing substance, wholly analogous to, if not identical with, the "anaphylactin" found in the serum of sensitized pigs by Gay and Southard. They state that: "It differs from whole horse serum and corresponds to the anaphylactin in the following particulars: (1) it is absolutely nontoxic for sensitive animals; (2) repeated large doses not only

¹ *Jour. Med. Res.*, 1908, 18, p. 407.

² *Jour. Exper. Med.*, 1908, 10, p. 1.

³ *Jour. Med. Res.*, 1908, 18, p. 433.

cause no refractory phase but abbreviate the period of incubation; (3) its injection into sensitive animals not only does not intoxicate, but also produces no refractory (antianaphylactic) phase; (4) it may give rise to sensitization of normal animals in a few days (four or five) as does the transfer of blood from a sensitive animal." They also observed that the ether-soluble constituents of serum, as well as the filtrate from the ammonium sulphate saturation, had neither sensitizing nor intoxicating properties.

These experiments are of particular importance as suggesting that the view that the sensitizing and intoxicating substances are the same, as originally maintained and repeatedly affirmed by Rosenau and Anderson, is not correct. They would seem to indicate rather that the sensitizing substance is either euglobulin itself or some closely related or adherent substance, while the toxic material is some more soluble protein, perhaps an albumin. On the other hand the studies of Vaughan and Wheeler¹ support the view that the sensitizing and the intoxicating substances are both parts of the same protein molecule, quite distinct from one another chemically but each possessing the specificity of the parent molecule. Their experiments were as follows: Egg-white is extracted with boiling absolute alcohol containing 2 per cent of NaOH, by which process the egg-white is split into a soluble poisonous fraction and a non-poisonous fraction which is insoluble in alcohol. The poisonous fraction gives all the protein reactions, except the Molisch test for carbohydrates, and although it is soluble in absolute alcohol Vaughan regards it as a protein on account of its giving all the color reactions. It might well be, however, that it is a soluble peptone or polypeptid containing enough of the different amino acids to give all the usual reactions, and the fact that it is evidently produced by hydrolysis of proteins is in support of this view. Be that as it may, this poisonous substance obtained from the proteins kills normal guinea-pigs with symptoms which, as described by Vaughan and Wheeler, are quite identical with those that appear so characteristically when sensitized guinea-pigs are killed by a second injection of the specific protein to which they have been sensitized. So typical are these symptoms that Vaughan develops the hypothesis that the anaphylaxis reaction is

¹ *Jour. Infect. Dis.*, 1907, 4, p. 476.

due in all cases to this very same poisonous element, which in sensitized animals is split out of the injected proteins by a specific enzyme which has been developed in the cells in response to the first injection of protein. The minimum fatal dose of this poisonous substance is small, "ranging from 8 to 10 or 100 mg. given intra-abdominally, according to the degree of purification reached." There is much disparity between the amount necessary to kill and the amount that suffices to produce symptoms. For example, with one preparation the fatal dose was 70 mg., but 5 mg. developed the earlier stages of intoxication in well-defined form. When given by mouth this poison is without ill effects.

The nontoxic fraction, which is insoluble in alcohol, also gives all the protein reactions and seems to contain all the carbohydrates of the original egg-white. This substance, although itself not poisonous, sensitizes to egg-white when given in doses as low as one or five milligrams, but does not sensitize to itself. This substance which produces sensitization is believed to be a protein, because when treated with uranyl acetate the filtrate left does not sensitize. Vaughan and Wheeler estimate that the second dose of egg-white must be large enough to liberate a fatal dose of poison on cleavage in order to produce death when injected into a sensitized guinea-pig; according to their experiments $\frac{1}{2}$ c.c. of filtered egg-white is not sufficient to cause a fatal effect, a result not in accord with the experience of others. The poisonous substance does not have the power of sensitizing to egg-white and thus is evidently free from the original sensitizing substance or from unbroken egg-white. Other proteins, including those from bacterial cells, have been found to yield these same poisonous and sensitizing substances, except that no sensitizing substance was obtained with casein or with the protein of the pneumococcus. In a later communication¹ it was stated that gelatin does not yield a poisonous substance, presumably because of its paucity in aromatic radicals. As Vaughan has not worked with pure proteins it cannot be said that these experiments conclusively prove that both sensitizing and poisonous elements are contained in one and the same protein molecule, for it is possible that the boiling with alkaline alcohol separates two different proteins, but the former conclusion seems much the more probably correct.

¹ Symposium on Immunity, American Association for the Advancement of Science, Chicago, Dec. 31, 1907.

ANAPHYLAXIS WITH PURE PROTEINS.

In developing the investigations of the chemistry of anaphylaxis, it seemed desirable, first of all, to discard the heterogeneous mixtures that have been commonly used in these studies, and, as the reaction is one concerning proteins, to work with the purest proteins obtainable that are suitable for the purpose. A number of the vegetable proteins can be obtained in a very pure condition, but these, for the most part, are not available for anaphylaxis studies because of their slight solubility in water. Rosenau and Anderson met with this difficulty when they attempted to sensitize with edestin and excelsin. As most suitable for the purpose, crystallized egg albumin was selected. The most successful way to secure a pure organic substance is, as a rule, crystallization and recrystallization. In egg albumin, from egg-white, we have a protein which crystallizes readily, for a protein, and which can be repeatedly recrystallized for purposes of purification; it also has the advantage of being readily obtained in large amounts, and numerous experiments have shown that crude egg-white is extremely active both in sensitizing and in intoxicating. Gay and Southard¹ found that the relative toxicity of egg-white, horse serum, and cow's milk is in a decreasing scale in the order given.

The crystallization was accomplished according to the method recommended by Hopkins² which gives good yields without great difficulty, if eggs but two or three days old are used and the process carried out carefully according to Hopkins' directions. The crystallized egg albumin was recrystallized two or more times, according to the purpose for which it was to be used, then dissolved in a minimum quantity of water, dialyzed until practically free from ammonium sulphate, dried in a current of air at room temperature, ground to a powder, and preserved in a desiccator for use. Prepared in this way the egg albumin may be kept indefinitely in an unchanging condition, and it is easily dissolved in water to form solutions of considerable concentration. Hopkins found that crystallized egg albumin after being recrystallized for a second time is a definite substance of uniform composition, and elementary analysis shows that the product obtained at the first recrystallization is composed of a pure substance,

¹ *Jour. Med. Res.*, 1908, 19, p. 5.

² *Jour. Physiol.*, 1900, 25, p. 306.

for it may be divided into several fractions by fractional recrystallization, each of which fractions gives the same analytic figures. Such a soluble, uniform, pure protein offers manifest advantages for the study of the anaphylaxis reaction, and it has been found to be, like whole egg-white, very active both in sensitizing and in intoxicating. In all the experiments with this substance it was used in a 5 per cent solution in distilled water, freshly dissolved for each day's use.

The minimum lethal dose was determined in pigs sensitized 19 days previously by intraperitoneal injection into pigs weighing about 300 gm. of 0.0005 gm. (0.01 c.c. of 5 per cent solution) of egg albumin that had been three times recrystallized. It was found that 0.0005 gm. of the same material given intraperitoneally in the second dose was usually fatal, and doses above this were invariably so. With old pigs the results were less constant, animals sometimes recovering from 0.005 gm., although made very sick by much smaller doses.

By the intravascular route even smaller doses were found to be toxic to sensitized pigs, as shown by the following experiment:

Four young pigs, weighing each 300 gm. were sensitized with 0.02 c.c. of 5 per cent solution of crystallized egg albumin. After 28 days they were given intracardiac injections of dilutions in salt solution of 5 per cent egg albumin solution, with the following results:

No. 1.—Received 0.002 c.c. 5 per cent egg albumin solution (0.0001 gm. of albumin) and showed severe typical symptoms in two minutes; after four minutes was apparently dead, then improved somewhat, but died after 20 minutes. (Autopsy showed that death had not been due to accidents of injection.)

No. 2.—Received 0.001 c.c. of solution (0.00005 gm. of egg albumin) and showed slight but typical symptoms after three minutes, but soon improved. After two hours gave 0.002 c.c. of solution (also intracardiac), which caused symptoms in two minutes and death in 30 minutes. (Autopsy excluded accidental death.)

No. 3.—Received 0.001 c.c. of solution, without any effects. One and one-half hours later gave 0.002 c.c., also without effect. Presumably this pig had not been sensitized.

No. 4.—Received 0.005 c.c. intraperitoneally without any observable effects. Three hours later 0.001 c.c. was given by intracardiac injection, causing symptoms in two minutes, and death in six minutes. (Autopsy excluded accidental death.)

The sensitizing dose of this same preparation is also extremely small, as shown by Table 1.

From this series it is seen that one-millionth of a cubic centimeter of 5 per cent solution of pure egg albumin, or *one-twentieth of a millionth of a gram of protein*, will sensitize a guinea-pig enough so

TABLE 1.

	AMOUNT OF SENSITIZING DOSE		SIZE OF SECOND DOSE*	INTERVAL BETWEEN INJECTIONS	RESULT
	5% sol.	Dry wt.			
1.....	0.5 c.c.	0.025 gm.	0.25 gm.	21 days	Death
2.....	0.05	0.0025	0.05	21 "	"
3.....	0.005	0.00025	0.025	21 "	"
4.....	0.0005	0.000025	0.2	21 "	"
5.....	0.00005	0.0000025	0.25	19 "	"
6.....	0.00002	0.000001	0.2	19 "	"
7.....	0.00001	0.0000005	0.2	19 "	Severe symptoms
8.....	0.000005	0.00000025	0.2	19 "	"
9.....	0.000002	0.0000001	0.2	19 "	Slight "
10.....	0.000001	0.00000005	0.2	19 "	Moderate "

* When not otherwise stated all injections were made into the peritoneal cavity.

that distinct and typical symptoms are produced after a second injection of the same material, while one-fifty-thousandth of a cubic centimeter of solution containing but *one-millionth of a gram of protein sensitizes fatally*.

Rosenau and Anderson¹ found that one-millionth of a cubic centimeter of horse serum will sometimes, but not always, sensitize, although in their series of experiments upon this point the smallest fatally sensitizing dose recorded was one-thousandth of a cubic centimeter. Gay and Adler² found that of their sensitizing proteid of serum (euglobulin) an amount corresponding to 0.01 c.c. of the original serum caused fatal sensitization, but sensitization with 0.0001 c.c. and 0.00001 c.c. caused symptoms without fatal results; however, they do not state just what quantity of euglobulin these figures represent. In any case, however, it would seem that the pure egg albumin is probably more actively sensitizing than the other substances that have been used in anaphylaxis studies.

The extremely minute quantities of crystallized egg albumin that were found effective in sensitizing and intoxicating, made comparison with unpurified egg-white desirable, and the following experiments were performed: Egg-white was diluted with an equal volume of water, beaten, and filtered. To a sample of this filtrate ten volumes of alcohol were added, the precipitated protein dried and weighed, and the solution was found to contain 6.3 per cent of protein. The minimum sensitizing dose of this solution was found to be (for 300-gram guinea-pigs) about 0.0001 c.c. (or 0.0000063 gm.). Doses smaller

¹ *Hygienic Laboratory Bulletin*, No. 29, 1906.

² *Jour. Med. Res.*, 1908, 18, p. 440.

than this produced no sensitization whatever, while fatal results were only obtained when 0.001 to 0.0005 c.c. were used for sensitizing.

The minimum intoxicating dose (intraperitoneal) of this solution, for pigs sensitized with unpurified egg-white, was found to be about 0.01 c.c. or 0.00063 gm.; but this amount produced but slight symptoms, fatal effects only resulting from doses as large as 0.04 c.c. or 0.0025 gm. of egg-white proteins.

Contrasted with the crystallized egg albumin, therefore, the unpurified mixed proteins of egg-white are about one-fifth as toxic for sensitive guinea-pigs, and but about one-hundreth as actively sensitizing.

This indicates that the toxicity of this purified protein is perhaps even greater than that of the whole egg-white, as if there might be inhibiting substances present in the untreated egg-white. The fatal dose of this protein, one-half of one milligram, is far smaller than the fatal dose of the poisonous fraction of egg-white prepared by Vaughan and Wheeler, which they give as "from 8 or 10 to 100 mg. given intra-abdominally, according to the degree of purification reached." They estimate that to liberate a fatal dose of their poisonous substance from egg-white, more than 60 mg. of the protein must undergo cleavage in the body, whereas these experiments show that one-half a milligram of pure protein furnishes a fatal dose for 300-gram pigs when given intraperitoneally and one-tenth as much is fatal when injected directly into the blood.

THE TOXIC ELEMENT OF PROTEINS.

These experiments may also be taken to indicate that both sensitization and intoxication may be produced by one and the same sort of protein molecule. Indeed, the sensitizing and intoxicating effects have been produced with such extremely minute quantities of protein that it seems not unwarranted to maintain that these experiments settle this point conclusively. As a repeatedly recrystallized egg albumin, giving every evidence of chemical purity, is capable of causing sensitization in doses of one twenty-millionth of a gram, and of killing sensitized pigs in doses of one-twentieth of a milligram, it is evident that if either of these effects were due to something other than the egg albumin itself, this contaminating substance must have

a potency beyond the bounds of the imagination. It is of course possible that in the case of horse serum the sensitization and the intoxication are effected by different proteins, as the work of Gay and Adler suggests—one cannot deny this possibility in the present incomplete state of our knowledge—but in the case of crystallized egg albumin such a possibility may be reasonably excluded.

Granting this, we have, among others, two possibilities—either the whole protein molecule accomplishes both the sensitization and the intoxication, or one part of the molecule does the sensitizing and another part of the complex structure produces the intoxication. Vaughan's work is, of course, in favor of the latter idea, for he seems to have been able to split out of impure proteins substances that will sensitize but not intoxicate, and substances that will intoxicate but not sensitize.

If the intoxication that follows the second injection of protein into animals that have been sensitized by a previous injection depends upon a cleavage of the proteins with liberation of toxic groups, we have several reasons for suspecting that the intoxicating element will be found to bear a close relation to the aromatic radicals of the protein molecule. In the first place we have the fact that compounds containing the benzene ring are commonly poisonous, from the simplest derivatives such as phenol and aniline up to complex substances such as the terpenes. Also it is known that in the digestion of proteins, bactericidal substances are formed, and these are apparently the derivatives of aromatic radicals of the proteins. Again, in their important studies upon the precipitins, which will be considered more extensively subsequently, Obermayer and Pick found that the specificity of the precipitin reaction seems to depend upon the aromatic radicals of the protein, an opinion supported by Fleischmann's observations on the effect of tryptic digestion upon serum. Finally, we have the statement of Vaughan that the toxic substance split off the proteins in his experiments, contains the aromatic radicals, and that gelatin, which is of all proteins the poorest in aromatic radicals, does not yield a toxic portion. In view of these facts it seems probable that gelatin should be defective in toxicity, and perhaps entirely unable to cause sensitization, and such has been found to be

the case.¹ Guinea-pigs were injected with doses of from 5 c.c. down to $\frac{1}{3\frac{1}{2}}$ c.c. of 4 per cent commercial gelatin solution, and after two weeks or more were given 8 to 10 c.c. of a similar solution intraperitoneally. In not a single case were any symptoms whatever produced by the second dose. Animals sensitized to egg-white or to bovine serum did not show any symptoms when injected with gelatin solution; and conversely, injection with gelatin did not sensitize to egg-white or bovine serum. Injection of gelatin solution into animals sensitized with egg-white did not make the animals at all refractory to injections of egg-white 24 hours later.

Although gelatin possesses of the aromatic compounds commonly found in proteins only a small quantity of phenyl alanine, and no tryptophane or tyrosine (probably), yet addition of tyrosine equal in amount to 6 per cent of the gelatin in a 4 per cent gelatin solution, did not produce a compound at all capable of causing either sensitization or intoxication, when used itself for both injections, or when used either as the first or second injection in connection with an injection of egg albumin or horse serum. This negative result was, of course, to be expected, for even if the toxicity of proteins depends upon their aromatic radicals these radicals probably act only in combination with many others in a large fragment of the entire protein molecule, and not as free radicals as in these experiments.

THE EFFECT OF HEAT UPON THE SUBSTANCE THAT IS CONCERNED IN ANAPHYLAXIS.

An objection that may be advanced against these experiments (one that Dr. J. F. Anderson has kindly made in a personal communication) is that gelatin is heated in its preparation, and heat destroys both sensitizing and intoxicating effects of proteins, at least those in serum. Concerning the effects of heat upon horse serum Anderson and Rosenau state in their latest publication² that "the sensitizing effect of horse serum is greatly influenced by heat and almost entirely disappears when the serum is heated to 100° C. for one hour." As to the effect of heat upon the intoxicating constituent, heating to 70° C. for one hour does not seem to diminish

¹ A preliminary report upon this topic has been published in the *Jour. Amer. Med. Assoc.*, 1908, 50, p. 527.

² *Jour. Med. Res.*, 1908, 19, pp. 38, 42.

appreciably its poisonous properties, but these seem to be somewhat affected at 80° C. for one hour. At 100° C. for one hour the toxicity apparently disappears. Gay and Adler found that the toxicity is decreased if the serum is heated at 90° for 15 minutes, and destroyed at 100° for 15 minutes; sensitization is accomplished by serum heated at 100° for 15 minutes, but not if heated 45 minutes. In order to prevent coagulation Rosenau and Anderson diluted the serum with three parts of distilled water, while Gay and Adler used nine parts of 0.85 per cent salt solution, but both note that at 100° the solution becomes more or less milky or opalescent.

Now while it cannot be denied that heat *may* in some way alter the gelatin molecule so that it cannot produce sensitization, yet there is very good reason to hold that the results obtained by heating horse serum cannot be applied to gelatin solutions, and that it is more probable that the inactivity of gelatin depends upon its chemical composition rather than upon the effect of the heat used in the preparation of gelatin from collagen by hydration. Gelatin is a member of a peculiar class of colloids forming "heat reversible gels;" that is, they are liquefied by heat and return to the gel form when cooled, which process can be repeated many times if no acids or alkalis are present to hydrolyze the gelatin. The application of heat up to 100° to gelatin, therefore, does not permanently change its physical properties, and it is just as capable of forming colloidal solutions as it was before. On the other hand, application to serum of temperatures considerably below the boiling point produces a severe physical change in the proteins by which they are coagulated and rendered insoluble; dilution of the serum does not alter this process except to prevent the coagulated particles from aggregating in large flocculi, for it does not require a very high power of the microscope to show that the opalescent heated serum dilution contains the protein in a granular, coagulated form. Now the behavior of such a protein when injected into the abdominal cavity is undoubtedly quite different from that of a protein that has not been coagulated. The uncoagulated protein is in a state of colloidal solution, and in a dissolved condition is absorbed unchanged, in evidence of which is the well-known fact that foreign proteins injected into the abdominal cavity of animals are excreted unchanged in the urine. This process of absorption should be quite the same with unheated serum and with gelatin solutions

which cannot be coagulated by heat. On the other hand, the situation with heat-coagulated proteins is quite different, for the protein does not then dissolve in the peritoneal fluids, but is, presumably, taken up like any other finely divided particles by the leucocytes and the endothelial cells, there to be digested and fundamentally altered before reaching the general circulation and the cells (whichever they may be) where the protein produces its effect. In other words, the fact that boiling destroys the sensitizing and intoxicating properties of heat-coagulable proteins is no evidence that it will affect in a similar way proteins that are not coagulable by heat, and hence the failure to secure sensitization or intoxication by gelatin is probably due not to the heating it has undergone, but rather is because of the chemical composition of the gelatin itself.

In support of the view that the effect of heat in interfering with the sensitizing and poisonous action of proteins depends entirely upon its effect in rendering the proteins insoluble, rather than by the production of chemical changes in the protein, may be cited the results of experiments with milk. The chief protein of milk, caseinogen, is not coagulated by heating to boiling, except for a small portion which dries out upon the surface to form the "skin" of scalded milk. As can be seen by the following experiments, heating milk to 100° for a considerable length of time does not destroy either its sensitizing or its intoxicating power.

TABLE 2.

	First Injection	Interval	Second Injection	Result
1.....	0.2 c.c. raw milk	21 days	5 c.c. raw milk	Death in 6 hours
2.....	0.2 " " "	21 "	10 c.c. milk (sour) heated 30 min. at 100° C. (partly curdled)	No symptoms. 24 hours later injected 5 c.c. raw milk causing only slight symptoms
3.....	0.2 " " "	21 "	10 c.c. fresh milk heated 30 min. at 100° C.	Death during night, after 6 hours
4.....	0.5 c.c. milk heated to 100° C., 5 min.	21 "	10 c.c. fresh milk heated to 100° C. for 40 min.	Death after 6 hours
5.....	0.5 c.c. milk heated to 100° C. for 25 min.	21 "	10 c.c. raw milk	Death in 15 min.
6.....	1.0 c.c. milk heated 45 min. to 100° C.	21 "	10 c.c. raw milk	Death in 6½ hour
7.....	1.0 c.c. raw milk	19 "	10 c.c. milk heated to 100° C. for 30 min.	Death in 75 min.
8.....	1 c.c. raw milk	19 "	10 c.c. milk heated to 100° for 40 min.	Slight symptom

A particularly interesting corroboration is furnished by experiment No. 2 of this series, in which the milk had become soured before being heated; the usual results of heating soured milk followed,

the caseinogen being converted into casein and precipitating out, and this milk alone lost its toxicity upon heating.¹ These experiments seem to show conclusively that whether a protein will be deprived of its toxicity by heating depends solely upon whether it is or is not coagulated by heat.

An additional bit of evidence pointing in the same direction is the following: Serum albumin, in contrast with egg albumin, can be permitted to stand for some time in strong alcohol by which it has been precipitated, without losing its solubility in water. If serum albumin is thus precipitated by alcohol and left standing a few hours, thoroughly washed with alcohol, dried and redissolved in water, it is found to be quite as actively toxic to sensitized guinea-pigs as fresh serum. On the other hand, if solutions of egg albumin are treated with alcohol in the same way, the albumin becomes almost entirely insoluble, and if it is washed free from alcohol, ground up to a fine emulsion in water, and injected into sensitized pigs, it produces no or very slight symptoms, and commonly it does not even render the animal refractory to a subsequent injection of egg albumin. This fact indicates, at least, that the mere process of coagulation of proteins does not seriously impair their toxicity, provided they are still soluble in water, and that the effect of coagulation of proteins upon their toxicity depends entirely upon the loss of solubility which the proteins suffer.

In this connection may be stated the results of experiments made to determine the effect of heat upon the sensitizing and intoxicating properties of pure egg albumin. On account of the apparent effect of coagulation upon the intoxicating action of serum and milk, these experiments were performed in pairs, to one of each of the pairs of heated albumin solution a drop of very dilute acetic acid being added after the heating was completed, to increase the degree of aggregation of the coagulated proteins. The results were as appear in Tables 3 and 4.

These experiments indicate that crystallized egg albumin is affected by heat in much the same way as is serum. The sensitizing power is not altogether destroyed at boiling temperature even with

¹ Apparently even with this curdled milk enough was absorbed, coagulation not having been absolutely complete, to render the animal refractory to an injection of raw milk 24 hours later.

TABLE 3.

EFFECT OF HEAT ON SENSITIZING POWER OF CRYSTALLIZED EGG ALBUMIN.

For each injection 5 c.c. of a 1 per cent solution of egg albumin, heated for 15 minutes at the temperature specified, was used. The second dose was given after an interval of 19 days, and consisted of 3 c.c. of unheated 5 per cent egg albumin solution.

Temperature		Symptoms in 10 minutes.			Results		
1.	100°				Death in 10 minutes.		
1A.	100°+acetic	"	" 20	"	never severe, and animal recovered.		
2.	90°				Death in 100 minutes.		
2A.	90°+acetic	"	" 23	"	but only slight and animal recovered.		
			" 36	"			
3.	80°				Death in 30 minutes.		
3A.	80°+acetic	"	" 10	"	" " 25 "		
			" 10	"			
4.	70°				" " 40 "		
4A.	70°+acetic	"	" 10	"	" " 28 "		
			" 10	"			

TABLE 4.

EFFECT OF HEAT ON INTOXICATING POWER OF CRYSTALLIZED EGG ALBUMIN.

The pigs had been sensitized 28 days previously with 0.2 c.c. of unheated egg albumin solution. They were injected with 2 c.c. of 5 per cent egg albumin solution diluted with 10 c.c. of water, which had been heated as indicated for 15 minutes.

Temperature		Symptoms in 22 minutes.			Results		
1.	90°				Slight or doubtful symptoms; recovered.		
1A.	90°+acetic				No symptoms.		
1B.	90°				Slight symptoms after 2 hours; recovered.		
2.	80°				Death in 4½ hours.		
2A.	80°+acetic	"	" 20	"	" " 2 "		
3.	70°				" " 25 minutes.		
3A.	70°+acetic	"	" 20	"	" " 45 "		
			" 10	"			

serum, provided large enough quantities of protein are injected, for I have obtained fatal sensitization of pigs with 2 c.c. of serum diluted to 10 c.c. with water, after heating to 100° for 45 minutes. This probably means either that minute quantities of protein escape coagulation through partial hydrolysis, or else that through the process of digestion of the coagulated material by the cells in the peritoneal cavity, a small quantity enters the blood in a soluble form still capable of sensitizing.¹ Toxicity, which requires a much larger dose to make it manifest, disappears at a lower temperature. Increasing the size of the coagulated particles, and perhaps also the

¹ It may be mentioned that in these experiments every necessary precaution was taken against introducing traces of unheated protein, which might cause sensitization in even minute quantities.

completeness of the coagulation by use of acetic acid, seems to augment slightly, but not very consistently, the effect of heat.

ANAPHYLAXIS WITH VEGETABLE PROTEINS.

In connection with the consideration of the influence of aromatic radicals of proteins upon their toxicity to sensitized animals, the alcohol-soluble vegetable proteins, zein and gliadin, were examined. The protein gliadin from wheat is, as compared with most proteins, poor in aromatic amino-acids, although it has much more than gelatin, while zein is particularly rich in tyrosine as is shown by the following table of results of hydrolysis of these proteins.

TABLE 5.

	Gliadin* per cent	Zein* per cent	Gelatin† per cent	Egg Albumin‡ per cent
Glycocoll.....	0.13	0	16.5	0
Alanine.....	1.33	2.23	0.8	2.1
Valine.....	?	0.29	present (?)	?
Leucine.....	6.30	18.60	2.1	6.1
Proline.....	9.82	6.53	5.2	2.25
Phenylalanine.....	2.70	4.87	0.4	4.4
Aspartic acid.....	0.25	1.41	0.56	1.5
Glutamic acid.....	33.81	18.28	0.88	8.0
Serine.....	0.06	0.57		
Tyrosine.....	1.19	3.55	0	1.1
Arginine.....	2.22	1.16	9.3	1.5
Lysine.....	0	0	5-6	
Histidine.....	0.39	0.43	0.4	
Tryptophane.....	present	0	0	present

* Osborne and Clapp, *Amer. Jour. Physiol.*, 1908, 20, p. 494.

† Fischer, Levene, and Aders, *Ztschr. physiol. Chem.*, 1902, 35, p. 70.

‡ Abderhalden and Pregl, *ibid.*, 1905, 46, p. 24.

The preparations of zein and gliadin were kindly furnished me by Dr. Thomas B. Osborne, and were thoroughly purified by reprecipitation. As they are not soluble in water, they were dissolved in 0.2 per cent NaOH solution to a concentration of 4 per cent of protein; this was then diluted as much as the exigencies of the experiment in hand warranted, commonly one-half, and NaHCO_3 added until signs of precipitation began, for the purpose of converting as much as possible of the NaOH into the less irritating Na_2CO_3 . The results of the experiments are given in Tables 6 and 7.

These experiments would seem to indicate that gliadin, with its low content of tyrosine and phenyl alanine, is weak in either intoxicating or sensitizing properties, presumably the former; however,

TABLE 6.
ZEIN ANAPHYLAXIS.

	First Injection	Interval	Second Injection	Results
1.....	0.04 gm.	18 days	0.12 gm.	Moderate symptoms; recovered
2.....	0.02	18 "	0.10	Severe symptoms; recovered
3.....	0.01	18 "	0.12	Severe symptoms; recovered
4.....	0.005	18 "	0.10	Slight symptoms; recovered
5.....	0.02	17 "	0.20	Death, 1 hour
6.....	0.004	17 "	0.25	Death, 40 minutes
7.....	0.002	18 "	0.20	Death, 12 hours
8.....	0.0004	17 "	0.20	Severe symptoms; recovered
9.....	0.0002	18 "	0.20	Severe symptoms; recovered
10.....	0.01	25 "	0.20	Death in 6 hours
11.....	0.01	25 "	0.20	Death in 6 (?) hours
12.....	0.005	25 "	0.15	Severe symptoms; recovered

NOTES.—The first four animals received for the second dose a solution of zein in 0.2 per cent of NaOH with a little chloroform, that had been standing in the ice chest for 18 days, which probably accounts for the less severe symptoms in this series. To make sure that the symptoms and death were not due to the alkalinity of the solution, control pigs received the same dose of each preparation used in these experiments, but only transient symptoms of peritoneal irritation were ever produced. Animals 8, 9, and 12 were each given a third injection of zein 24 hours after the intoxicating dose, but in none were symptoms observed, showing that zein produces a refractory stage in animals recovering from intoxication. In all experiments the onset of symptoms was rather slow, as compared with serum sensitization which is presumably due to the relatively poor solubility of zein in the body fluids.

TABLE 7.
GLIADIN ANAPHYLAXIS.

	First Injection	Interval	Second Injection	Results
1.....	0.04 gm.	18 days	0.20 gm.	Severe symptoms; recovered
2.....	0.01	19 "	0.20	" " "
3.....	0.005	17 "	0.10	Slight " "
4.....	0.001	18 "	0.12	" " "
5.....	0.02	19 "	0.20	Moderate " "
6.....	0.02	19 "	0.20	" " "
7.....	0.02	20 "	0.20	" " "
8.....	0.01	14 "	0.20	" " "
9.....	0.004	14 "	0.20	No symptoms
10.....	0.01	14 "	0.16	No symptoms
11.....	0.2	23 "	0.20	Died, 45 minutes
12.....	0.2	23 "	0.20	Moderate symptoms; recovered
13.....	0.04	14 "	0.20	No symptoms
14.....	0.02	14 "	0.20	No symptoms
15.....	0.2	20 "	0.25	No symptoms
16.....	0.2	20 "	0.25	No symptoms
17.....	0.004	26 "	0.25	Slight symptoms; recovered
18.....	0.02	26 "	0.25	" " "
19.....	0.08	26 "	0.25	" " "
20.....	0.16	26 "	0.25	" " "

the preparation of gliadin used was so readily thrown out of solution that it is possible that part of this apparent inertness is due to insolubility, although I do not consider this probable. With zein there was no difficulty in securing effective sensitization and fatal intoxication with all the characteristic symptoms, and the zein is little more soluble than the gliadin. This action of zein is particularly inter-

esting in view of the absence of tryptophane from the zein molecule, and would seem to indicate that this aromatic radical, at least, is not essential for intoxication by proteins.

Other experiments showed that zein does not sensitize to gliadin, and conversely. This was to be expected in view of the fact that with the precipitin reactions the vegetable proteins show a species specificity fully as sharp as is observed among animal proteins, and according to Gasis¹ even more distinct.

Although all the experiments described above, together with some facts to be brought out later, seem to point to the aromatic radicals of the protein molecule as being in some way essentially concerned in the intoxicating effects which proteins exert upon sensitized guinea-pigs, yet it is rather difficult to believe that it is actually the aromatic radicals, or for that matter any other part of the protein molecules that are injected into the guinea-pig for the second dose, that act as the fatal poison. If we consider the quantity of pure protein that is sufficient to kill a sensitized guinea-pig, but 0.00005 to 0.0001 gm., and try to imagine what could be derived from that amount of protein that would produce fatal intoxication, we certainly find it a difficult problem. If there is a poisonous group which is liberated from the second dose of protein, whether by a special enzyme as Vaughan suggests or by some other process, it must be something most wonderfully poisonous, and it is very difficult to understand from what we know of the structure of the protein molecule just how it could produce so exquisite a poison and what the structure of this remarkable poison might be. Of course we may assume that under the special conditions of sensitization the cells become peculiarly permeable, or have an especial affinity for the unknown toxic substances, so that we cannot compare them with the ordinary poisons which act in animals not specially sensitized to them. Or we may perhaps legitimately imagine that the second dose injected into sensitized guinea-pigs causes their cells to produce from their own constituents or from the constituents of the blood the poisons that produce the fatal results. As an analogy for this latter hypothesis we have the fact that when a serum containing specific precipitins is added to a small quantity of the homologous protein the precipi-

¹ *Berl. klin. Wchnschr.*, 1908, 45, p. 358.

tate that forms is derived chiefly from components of the specific serum and not from the protein with which it reacts.

However, the rapidity with which the intoxication occurs once the second dose of serum has entered the blood, oftentimes but a fraction of a minute after intracardiac injections, speaks somewhat against the idea that any complicated process has to go on before the toxic substance is prepared, and consequently is in favor of the view that it really is the protein itself or some substance that can be readily formed from it that exerts the lethal effect. Much more evidence must be secured before we can determine this point.

EFFECT OF IODIZATION OF PROTEINS.

At the present time the determined facts concerning the anaphylaxis reaction indicate that it may be something quite different from the known reactions of immunity, but in its limitation to the protein substances it has much in common with them, and in the fact that specific reactions can be produced with a great variety of proteins it recalls in particular the precipitin reaction. In connection with the problems of the precipitin reaction, Obermayer and Pick¹ have made interesting and important observations, which may be briefly summarized as follows: The proteins seem to contain two fundamental groupings of amino-acid radicals, one of which determines the specificity of the precipitin reaction for the species of animal from which the protein comes; and another *konstitutive Gruppierung* which determines the specificity that is characteristic for the protein itself and which varies with the total structure of the entire protein molecule. The former of these, the species specificity, is much more firmly established, and resists alterations in the molecule much longer than the latter. To illustrate, if an animal is immunized to normal bovine serum its serum will not precipitate bovine serum that has been altered by heat. However, if immunization is done with heated bovine serum, the animal's serum will react not only with heated bovine serum, but also with normal bovine serum and with a whole series of its splitting products with which the serum of animals immunized to normal bovine serum will not react. But the serum against heated bovine serum is just as specific as the other,

¹ *Wien. klin. Wchnschr.*, 1906, 14, p. 327.

in respect to species, for it will react only with proteins of bovine origin. Similar effects may be produced by altering proteins in various ways, such as converting them into alkali- or acid-albuminates, or acting upon them with formaldehyde, toluol for a long period of time, or by partial oxidation with alkaline permanganate solution. In other words, it is very easy to alter a protein so that it produces precipitins for proteins other than itself, provided these proteins come from the same animal species as it comes from itself. On the other hand, the specificity for species seems to be affected only when the aromatic radicals of the protein molecule are acted upon in various ways. This is most readily done by introducing such radicals as iodine, NO_2 , and $\text{N}=\text{N}$, into the protein molecule, where they are known to enter specifically the benzene rings of the aromatic radical. It was found that such altered proteins produce precipitins that are specific for the form of protein used in immunizing, but not at all specific as regards the species from which the protein is derived; e. g., iodized bovine serum produces in immunized rabbits precipitins that will react only with iodized proteins, but they react with iodized proteins of any source, whether from egg-white, horse serum, or serum of some other animal, even of iodized rabbit proteins. On the other hand, such modified proteins are quite specific toward themselves, that is, precipitins for iodized proteins will not react with diazotized proteins, and conversely. Finally, if radicals are introduced into amino-acids other than those entered by iodine, etc. the species specificity is not lost; e. g., diazo-benzol radicals enter the protein molecule, probably into the histidine group, and the resulting diazobenzol-protein produces precipitins specific for itself and which also exhibit specificity for the particular animal species from which the protein was derived.

These important observations have not yet, so far as I can learn, been subjected to either re-examination or to extension, and it seemed highly desirable to learn to what extent they might apply in the anaphylaxis reaction. For this purpose the following experiments were performed:

Iodized serum, Preparation I.—Preparation I of iodized bovine serum was prepared according to the procedure recommended by Blum,¹ as follows: To 50 c.c.

¹ *Ztschr. physiol. Chem.*, 1899, 28, p. 288.

bovine serum was added 1.5 gm. KI, 0.75 gm. iodine, and 0.05 gm. potassium iodate, and the mixture was made faintly alkaline with 2 c.c. of 10 per cent sodium bicarbonate solution. Kept in water bath at 45° for two hours, then at 38° for 18 hours; then added one-fifth as much of the I-KI solution as first used, and heated at 45° for one hour. There was a small amount of precipitate, which was removed by filtration, and the clear filtrate was dialyzed through collodion against cold running water for 48 hours. Analysis of the iodized serum solution left in the dialyzer showed it to contain 4.24 per cent by weight of protein coagulable by alcohol (dilution through endosmosis had occurred), and this protein contained 8.89 per cent of iodine. As completely iodized serum albumin contains 12 per cent of iodine by weight (Kurajeff),¹ and serum globulin contains 13 to 14 per cent (Hopkins) it is evident that this preparation represents an incompletely saturated iodo-protein. It also differs from the typical iodo-proteins in being soluble in water, and in giving Millon's reaction, but resembles them in that the iodine is so firmly bound that it cannot be detected by the usual reactions.

With this preparation the following results were obtained:

TABLE 8.

	First Injection	Interval	Second Injection	Results
1.....	5 c.c. iodized serum	16 days	5 c.c. iodized serum	Death in 45 min.
2.....	0.01 " "	16 "	5 " "	Severe symptoms
3.....	0.02 " "	24 "	7 " "	Death in 90 min.
4.....	0.02 " "	30 "	7 " "	Death in 20 min.
5.....	0.1 " "	16 "	5 bovine serum	No definite symptoms
6.....	0.001 " "	16 "	5 " "	Death in 35 min.
7.....	0.02 " "	30 "	6 " "	Death in 30 min.
8.....	0.02 " "	24 "	5 " "	Severe symptoms
9.....	0.02 " "	21 "	1 egg-white	No symptoms
10.....	0.02 " "	60 "	0.25 gm. egg albumin	No symptoms
11.....	0.02 " "	21 "	0.2 gm. zein	No symptoms
12.....	0.02 " "	21 "	3 c.c. dog serum	Slight symptoms
13.....	0.02 " "	18 "	3 " "	No symptoms
14.....	0.02 " "	19 "	5 " "	No symptoms
15.....	0.02 " "	18 "	3 horse serum	No symptoms
16.....	0.05 bovine serum	21 "	5 iodized serum	No symptoms
17.....	0.05 " "	22 "	5 " "	No symptoms
18.....	0.05 " "	23 "	5 " "	No symptoms
19.....	0.05 " "	29 "	5 " "	No symptoms
20.....	0.01 " "	21 "	5 " "	Death in 50 min.

These results indicate that this particular incompletely iodized serum possesses the property of fatally sensitizing guinea-pigs to normal bovine serum and to itself, but it does not sensitize to foreign proteins. What is of particular interest is that it does not intoxicate pigs sensitized with normal bovine serum (only one exception observed), although quickly killing pigs sensitized to itself. The pigs that failed to react to the iodized serum (Nos. 16-19) were given 5 c.c. each of normal bovine serum 24 hours later and all reacted distinctly (two moderately, two severely) but recovered, indicating that the iodized serum had not conferred the refractory phase commonly

¹ Mann, *Chemistry of the Proteins*, p. 231.

seen in animals recovering from an intoxicating dose, although the animals were perhaps somewhat protected by the iodized serum since none of them died. The animals sensitized with iodized serum that recovered from injections of heterologous proteins (Nos. 9 to 15), were given 24 hours later normal bovine serum, and all developed more or less severe symptoms, only one (No. 14), however, dying.

Iodized serum, Preparation II.—200 c.c. bovine serum, which contained considerable hemoglobin, was treated as in the previous preparation, except that the temperature at one time rose to 50°. There was a large amount of precipitate, which was, unfortunately, not saved, only the filtrate being dialyzed. The dialyzed filtrate represented a dilute protein solution, the ammonia from 5 c.c. decomposed by the Kjeldahl method requiring but 7 c.c. $n/4$ acid to neutralize, as against 19 c.c. required by the original serum. Analysis showed it to contain but 2.8 per cent of protein coagulable by alcohol, and this protein contained only 6.3 per cent of iodine. Evidently, therefore, a large proportion of this protein has been lost by precipitation, and the soluble remainder is but about one-half saturated with iodine—even less than Preparation I. The results of experiments with this preparation follow:

TABLE 9.

	First Injection	Interval	Second Injection	Results
1.....	0.02 c.c. bovine serum	32 days	5 c.c. iodized serum II	Death in 45 min.
2.....	0.02 iodized serum I	24 "	7 " "	Death in 45 min.
3.....	0.02 " " I	29 "	6 " "	Death in 30 min.
4.....	0.02 " " I	53 "	5 " "	Death in 55 min.
5.....	0.05 bovine serum	49 "	6 " "	Severe symptoms; recovered
6.....	0.01 " "	19 "	4 " "	Death in 55 min.
7.....	0.01 " "	19 "	3 " "	Death in 140 min.

This preparation of iodized serum differs from the first preparation in the fact that it intoxicates guinea-pigs sensitized to normal serum equally as well as those sensitized to iodized serum.

Iodized serum, Preparation III.—On account of this disparity of results a third preparation of bovine serum was prepared, which resembled in all respects Preparation No. II. The heavy precipitate obtained from the serum was also collected and washed. It differed from iodized proteins generally in being but very slightly soluble in dilute (0.1 per cent) NaOH, and not at all in Na_2CO_3 (1 per cent). Analysis of the insoluble residue showed it to contain 5.9 per cent of iodine, while the serum filtered from it, after dialysis, contained but 1.3 per cent of protein, and this protein contained but 1.88 per cent of iodine. Why these three preparations should differ so much from one another is not known, for all were prepared alike. The soluble part of the third preparation was found to have the same properties as Preparation II, and no further experiments were made along this line.

Rosenau and Anderson¹ in their latest publication make the following statement concerning the influence of iodine on serum, in

¹ *Jour. Med. Res.*, 1908, 19, p. 40.

respect to anaphylaxis: "We made several tests to determine the effect of iodine upon the toxic action of horse serum and it so turned out in the preliminary experiments that the symptoms appeared to be profoundly modified in the sense that they were either delayed or inhibited. We therefore tested a large number of guinea-pigs to determine this point but found that so far as the toxicity of horse serum is concerned at the second injection, it was not appreciably modified by the iodine. The iodine was added to horse serum in the proportion of 1.5 grams per twenty-five cubic centimeters of serum and three grams of potassium iodide to aid solution." Regarding the irregularity of the results obtained by Rosenau and Anderson, my own experiments show indications of similar irregularity, but in the case of iodized serum this is probably due to the incompleteness of the iodization as shown by analysis. The details of Rosenau and Anderson's experiments as published do not indicate that they controlled the completeness of their iodization by analysis.¹

IODIZED EGG ALBUMIN.

Although such unreliable results were obtained with the iodization of serum, it was hoped that more satisfactory preparations might be obtained with pure proteins, and this was found to be the case. Using the pure egg albumin, recrystallized three times, and proceeding as with the iodization of the serum, an iodized protein was precipitated out in the dialyzing sac which seems to be a saturated iodized egg albumin. It contains 8.97 per cent of iodine,² agreeing well with Hofmeister's preparation of iodized egg albumin, which contained 8.9 per cent of iodine. It is quite insoluble in water, but dissolves slowly in 1 per cent Na_2CO_3 , and readily in 0.1 per cent NaOH . The solution in dilute NaOH is not thrown down by NaHCO_3 , but is by acetic acid, redissolving readily when the solution is made alkaline. The tyrosine is so well saturated that the protein does not give Millon's reaction, even when heated once to boiling; it does, however, give the Hopkins-Cole reaction for tryptophane. Therefore in all respects this preparation seems to be a typical saturated iodo-albumin.

In the experiments recorded below the iodo-protein was used in 5 per cent solution, dissolved in 0.1 per cent NaOH , to which was

¹ *Hygienic Laboratory Bulletin*, No. 45, 1908.

² For all the determinations of iodine I am indebted to Mr. Conrad Jacobson.

then added the calculated amount of NaHCO_3 to convert all the sodium hydroxide into the less irritating sodium carbonate.

TABLE 10.

	First Injection	Interval	Second Injection	Results
1.....	0.001 gm. egg albumin	20 days	0.12 gm. iodized albumin	Severe symptoms
2.....	0.001 " "	18 "	0.15 " "	Death in 6 min.
3.....	0.1 " "	30 "	0.20 " "	Severe symptoms
4.....	0.1 " "	30 "	0.20 " "	Moderate symptoms
5.....	0.005 iodized alb.	23 "	0.15 " "	Slight symptoms
6.....	0.005 " "	23 "	0.15 " "	Slight symptoms
7.....	0.005 " "	23 "	0.15 " "	Severe symptoms
8.....	0.003 " "	23 "	0.13 " "	Death in 80 min.
9.....	0.0005 " "	23 "	0.13 " "	Death in 8 hours
10.....	0.0005 " "	26 "	0.20 egg albumin	No symptoms
11.....	0.005 " "	26 "	0.20 " "	No symptoms
12.....	0.005 " "	23 "	0.20 " "	Severe symptoms
13.....	0.005 " "	22 "	4.0 c.c. bovine serum	No symptoms
14.....	0.005 " "	22 "	5 dog serum	No symptoms
15.....	0.005 iodized serum I	17 "	0.15 gm. iodized alb.	No symptoms
16.....	0.05 " " I	21 "	0.15 " "	No symptoms
17.....	0.05 dog serum	17 "	0.15 " "	No symptoms
18.....	0.05 " "	21 "	0.15 " "	No symptoms

These experiments all indicate that the iodization of egg albumin and of serum does not have the same effect upon the specificity of the anaphylaxis reaction, that it was found to have upon the precipitin reaction by Obermayer and Pick. Iodized albumin does not sensitize to heterologous proteins, nor do heterologous proteins, even when iodized, sensitize to iodized egg albumin. Apparently iodized albumin does not effectively sensitize to the pure crystallized albumin, and it is not so effectively intoxicating to pigs sensitized with iodized protein as pure albumin is to pigs sensitized with egg albumin. Apparently, therefore, the saturation of the aromatic radicals with iodine decreases the toxicity of the proteins, and it also impairs their power of sensitizing animals to the original non-iodized protein. However, the somewhat impaired solubility of the iodized protein may here also play a part.

ACTION OF TRYPSIN UPON SERUM.

As one of the most characteristic features of proteins is their disintegration by proteolytic enzymes, and particularly as these enzymes affect nothing else than proteins, a very essential step in proving the protein nature of the bodies concerned in anaphylaxis is the determination of the effect of digesting solutions containing them with trypsin or pepsin or both. So far nothing positive has been

reported concerning this question, for the experiment of Rosenau and Anderson, who let serum stand over night at 15° with pancreatin, is of necessity devoid of significance, since under these conditions trypsin could have no appreciable effect upon the proteins of the serum. Unheated serum possesses such a resistance to the action of trypsin that even at 38° digestion proceeds very slowly for the first day, and any extensive disorganization of the serum proteins is a matter of months, as will be shown in the experiments to be cited below. In connection with their studies upon the chemistry of the specificity of proteins as shown by precipitin reactions, Obermayer and Pick investigated the effects of tryptic digestion upon serum and egg-white. They found that if the digestion was allowed to continue until the disappearance of the biuret reaction, the resulting material when injected into rabbits caused the development of precipitins which reacted only upon the same digestion mixture that was used in immunizing; this precipitin was, in spite of the proteolytic disintegration of the precipitogenous material, specific for the species furnishing the latter; i. e., precipitins produced by digested bovine serum will not react with digested horse serum, and conversely. Therefore, in spite of the extensive splitting of the proteins, the group upon which the specificity for species depends is intact even when the biuret grouping itself has been largely destroyed. Other experiments have indicated that digestion with pepsin-HCl causes the disappearance of precipitogens by the time all the coagulable proteins have disappeared, therefore much earlier than with tryptic digestion; also, a preparation obtained by digesting beef pancreas until biuret free did not produce precipitins.

Fleischmann,¹ who has also studied the effect of digestion upon the precipitin reaction, reaches results that are not in harmony with those obtained by Obermayer and Pick. He found that serum digested with trypsin loses its precipitability somewhat before the material ceases to give the usual protein reactions, while peptic digestion causes a similar condition in serum when but two-thirds of the coagulable protein has disappeared. He found that after about five months of tryptic digestion of a specimen of bovine serum it had lost its biuret reaction, and it was then used to immunize two rabbits.

¹ *Ztschr. f. klin. Med.*, 1906, 59, p. 515.

They were given 12 c.c. doses at intervals of 14 days, and one rabbit died after the second injection (probably it had been sensitized, for the conditions were ideal for such a result). The surviving rabbit showed no precipitin after the third injection, but after the fourth injection its serum contained a precipitin which precipitated heterologous proteins, such as goat and sheep serum and egg-white, and antibodies against human and rabbit serum were also demonstrated by fixation of complement. Evidently, therefore, sufficient digestion of a protein will destroy its specificity as indicated by the precipitin reaction. Fleischmann tabulates the results obtained by others who have investigated the effects of digestion upon the precipitogens of serum and finds marked discrepancies in the reports of different experimenters. These variations in results he ascribed to the variation in activity of different digestion mixtures.

EFFECT OF TRYPTIC DIGESTION UPON PROTEINS.

In connection with these results, and with those to be given below, should be considered the nature of the changes brought about in the protein molecule by the action upon it of trypsin. It has long been known that the process of proteolysis is not completed through a sudden collapse of the protein molecule, but rather it goes on step by step, with a gradual decrease in size of the molecule, so that at first we have the comparatively large proteose molecules and then the somewhat smaller peptone molecules, and finally the single amino-acid molecules are found free and the entire molecule of the protein has been converted into diffusible crystalloidal substances. The exact nature of these changes has been considerably cleared up of late by the investigations that are being carried on in the laboratory of Emil Fischer, and may be briefly summarized as follows:¹

The first effect of the trypsin on the protein is to split out the tyrosine radicals, so that in a short time practically all the tyrosine is free in the digestion mixture, although the protein molecule is far from completely disintegrated. Tryptophane is also split out early, and likewise the cystine, while the other amino-acids hold together longer. For example, Abderhalden and Reinbold found that when edestin was digested with trypsin, after 24 hours 78.4 per cent of the tyrosine

¹ See Emil Abderhalden, *Lehrbuch der physiologischen Chemie*, Berlin, 1906, p. 179.

had been liberated, while of the glutamic acid but 4.3 per cent had been split out; after two days 97.6 per cent of the tyrosine as contrasted with 7.4 per cent of the glutamic acid had been liberated, and while all the tryosine had been split out after 8 days, at this time but 31.1 per cent of the glutamic aid was free. Of the other amino-acids, alanine, leucine, amino-valerianic acid, and aspartic acid behave about the same as the glutamic acid. On the other hand, after very extensive digestion of proteins no free phenylalanine or proline can be found, for these radicals remain obstinately united, forming polypeptids which consist chiefly of these two amino-acids associated with small quantities of the other amino-acids exclusive of tyrosine, tryptophane, and cystine. If the protein contains glyco-coll this can commonly be demonstrated to resist cleavage along with the phenylalanine and proline.

It is evident, therefore, that the changes taking place in serum or other proteins undergoing tryptic digestion are such as tend to eliminate the tyrosine and tryptophane, leaving as the representative of the aromatic radicals the phenylalanine, which the gelatin experiments suggest is perhaps not a conspicuous factor in the intoxication of sensitized guinea-pigs. Fleischmann's observation that the specificity of proteins is impaired under the influence of protracted tryptic digestion, in which presumably most of the tyrosine and tryptophane have been removed, is in harmony with the claim of Obermayer and Pick that specificity depends upon the aromatic groups. However, the value of these observations is largely nullified by their lack of agreement, and also by the fact that one of the aromatic radicals, the phenylalanine, remains to the last with the residue of the protein molecules.

EFFECTS OF TRYPTIC DIGESTION OF BOVINE SERUM UPON ITS SENSITIZING AND INTOXICATING PROPERTIES.

The digestion of a specimen of bovine serum with trypsin was begun on February 28, 1908. To 750 c.c. of serum, which had been kept with a little chloroform for a few days in the refrigerator, was added 7.5 gm. of commercial pancreatin (Parke, Davis & Co.). This mixture was analyzed and found to contain per cubic centimeter 13.6 mg. of nitrogen, of which 90 per cent was coagulated by heat

and 10 per cent non-coagulable. There was then added 0.75 gm. Na_2CO_3 and 10 c.c. chloroform, and the mixture was placed in a thermostat at 37.5° , being occasionally shaken.

After 10 days digestion (March 9) a sample was analyzed, and now but 22.7 per cent of the nitrogen was found to be coagulable, while 77.3 per cent was non-coagulable. The coagulum, after repeatedly washing with hot water, was found to still give both the Millon reaction for tyrosine and the Hopkins-Cole reaction for tryptophane, showing that these substances had not been entirely split out of the coagulable proteins. A part of the serum was set aside and labeled "Digestion Mixture I," and the remainder was returned to the incubator.

The results obtained with the digested serum¹ at this stage are shown in the following table:

TABLE 11.

	First Injection	Interval	Second Injection	Results
1....	6 c.c. digest. mixture	24 days	2 c.c. digestion mixture	Slight symptoms; recovered
2....	0.5 " "	25 "	3 " "	No symptoms
3....	0.1 " "	24 "	3 bovine serum	Death in 75 min.
4....	0.01 " "	24 "	2.5 " "	Very severe symptoms; recovered
5....	0.1 " "	24 "	2 digestion mixture	Slight symptoms; recovered
6....	0.01 " "	25 "	4.5 " "	Slight symptoms; recovered
7....	0.05 bovine serum	20 "	4 " "	Slight symptoms; recovered

The five pigs that recovered were each given 3 c.c. bovine serum on the next day, and showed slight or no symptoms, indicating that the second injection of digestion mixture, although not causing serious symptoms, had produced a refractory period such as follows recovery from intoxicating doses generally.

The results of this series of experiments may be summarized as follows:

After tryptic digestion of bovine serum until but 22.7 per cent of the nitrogen is in the form of coagulable proteins, the toxicity (intraperitoneally) for sensitized pigs is almost entirely destroyed, although the sensitizing power for bovine serum remains, at least for sensitizing doses of 0.01 c.c. or more. Recovery from a second large dose of digestion mixture renders the animal refractory to bovine serum.

¹ In these and all other experiments in which material was used that had been preserved with chloroform, the material was freed from chloroform by placing in a shallow dish in a vacuum chamber for a short time before using it for injection.

After the digestion had continued 21 days there was a heavy precipitate in the mixture. A sample was filtered, and the filtrate analyzed. There had been some loss of nitrogen, presumably in part through precipitation and in part from escape of ammonia, for each cubic centimeter of filtrate contained 12.2 mg. nitrogen as against 14.6 mg. per cubic centimeter at the beginning. Of this nitrogen but 8 per cent was coagulable, and 92 per cent was not. The coagulum, however, still contained tyrosine and tryptophane as shown by appropriate tests. A portion of the digestion mixture, labeled "Digestion Mixture II," was reserved for experiments, and the remainder was returned to the incubator.

This preparation yielded the following results:

TABLE 12.

	First Injection	Interval	Second Injection	Results
1...	5 c.c. Digest. Mixt. II	21 days	5 c.c. bovine serum	Moderate symptoms; recovered
2...	0.25 " " "	21 "	5 Digestion Mixture	No symptoms
3...	0.25 " " "	21 "	5 horse serum	No symptoms
4...	0.04 " " "	21 "	5 bovine serum	Death in 35 min.
5...	0.04 " " "	21 "	10 milk	No symptoms
6...	0.004 " " "	21 "	5 bovine serum	Severe symptoms; recovered
7...	0.004 " " "	21 "	5 Digestion Mixture	No symptoms
8...	0.0004 " " "	34 "	5 bovine serum	No symptoms
9...	0.0004 " " "	21 "	5 " "	No symptoms
10...	0.05 bovine serum	18 "	5 Digestion Mixture	No symptoms
11...	0.05 " " "	18 "	5 " "	No symptoms

Twelve days after the second injection a third injection was given each pig (except Nos. 4 and 8), this time consisting of 3 c.c. of bovine serum in each case. The results were as follows: (1) Second injection was bovine serum. Developed slight symptoms, but never became very sick; (2) Second injection was digestion mixture. Became very sick in 15 minutes and nearly died, but recovered eventually; (3) Second injection was horse serum. Died in 75 minutes; (5) Second injection was milk. Died in 70 minutes; (6) Second injection was bovine serum. Showed only slight symptoms; (7) Second injection was digestion mixture. Died in between two and four hours; (9) Second injection was bovine serum. Showed only slight or doubtful symptoms.

The results of this series may be summarized as follows: Digestion of serum by trypsin until but 8 per cent of the nitrogen exists as coagulable protein destroys, or at least greatly reduces the poisonous action on sensitized pigs, so that 5 c.c. intra-abdominally is without effect. This digestion mixture, however, sensitizes in doses of 0.004 c.c. but not in doses of 0.0004 c.c., as contrasted with normal serum which sensitizes uniformly in doses of 0.0001 c.c. and usually in doses of 0.00001 c.c. It does not intoxicate animals sensitized to

itself, or to unaltered serum, and the second large dose of digestion mixture does not make the animals refractory to bovine serum given 12 days later, although if the second dose is bovine serum the animals are refractory. The specificity of the serum has not been destroyed, for animals sensitized to digested bovine serum do not react to horse serum or milk, and are not rendered refractory by these substances.

After 59 days digestion another sample was removed for study, the remainder was filtered to remove the heavy precipitate, and 1 gm. pancreatin and 5 c.c. chloroform were added, and it was returned to the incubator. The sample, after filtration, contains 4.3 per cent of its nitrogen in coagulable forms and 95.7 per cent not coagulable and the coagulated protein still gives good reactions for tyrosine and tryptophane, the filtrate giving a strong biuret reaction. This material, designated as "Digestion Mixture III," was used in the following experiments:

TABLE 13.

	First Injection	Interval	Second Injection	Results
1...	0.1 c.c. Digest. Mixt. III	28 days	3 c.c. bovine serum	Death in 22 min.
2...	0.02 " " "	23 "	3 " "	Death in 60 min.
3...	0.01 " " "	23 "	3 " "	No symptoms
4...	0.005 " " "	23 "	3 " "	No symptoms
5...	0.002 " " "	28 "	3 " "	No definite symptoms
6...	0.001 " " "	28 "	4 " "	No definite symptoms
7...	0.0005 " " "	28 "	4 " "	No symptoms
8...	0.0002 " " "	28 "	4 " "	No symptoms
9...	1.0 " " "	28 "	6 Digest. Mixt. III	Death in 1 hour
10...	0.02 " " "	28 "	5 " " "	No definite symptoms
11...	0.16 " " "	22 "	5 " " "	Death in 2 hours
12...	0.05 bovine serum	16 "	5 " " "	No symptoms
13...	0.16 Digest. Mixt. III	21 "	0.2 gm. zein	No symptoms
14...	0.16 " " "	21 "	5 c.c. dog serum	No symptoms
15...	0.16 " " "	21 "	5 horse serum	No symptoms
16...	0.16 " " "	21 "	0.2 gm. egg albumin	No symptoms
17...	0.16 " " "	31 "	1 c.c. Digestion Mixture (intracardiac)	Death in 1 min.
18...	0.16 " " "	22 "	1 " " "	Severe symptoms; recovered
19...	0.16 " " "	22 "	1 " " "	Death in 4 min.
20...	0.01 bovine serum	28 "	1 " " "	Death in 4 min.
21...	0.1 " " "	35 "	1 " " "	No symptoms
22...	0.1 " " "	35 "	1 " " "	No symptoms
23...	0.05 gm. gliadin	30 "	1 " " "	No symptoms
24...	0.05 " " "	30 "	1 " " "	No symptoms
25...	0.05 zein	30 "	1 " " "	No symptoms

These experiments show that by tryptic digestion the sensitizing power of serum is steadily reduced, so that after 59 days digestion of this sample (when but 4.7 per cent of the nitrogen is coagulable), the minimum sensitizing dose to undigested serum is one-fiftieth of a cubic centimeter as contrasted with normal serum which sensitizes in doses of one-hundred-thousandth cubic centimeter or less.

On the other hand, the toxicity is so reduced that 5 c.c. intraperitoneally or 1 c.c. by intracardiac injection do not ordinarily produce symptoms in guinea-pigs sensitized to bovine serum. However, pigs sensitized to the digestion mixture died with typical symptoms when injected with 5 or 6 c.c. of the same intraperitoneally, and pigs with the same sensitization usually died when given 1 c.c. by the intracardiac route, whereas normal pigs treated in the same way showed no effects. Possibly this indicates a resemblance to some of the features of Obermayer and Pick's results on immunization with serum that had been digested with trypsin, for they found that it produced precipitins reacting with the digested serum but not with undigested serum. The extensive digestion of this experiment seems not to have at all impaired the specificity of the serum for it does not sensitize to zein, egg albumin, horse serum, or dog serum. Pigs sensitized with heterogenous proteins, and usually also even those injected with normal bovine serum, were not sensitized to the digestion mixture.

On July 19, 1908, after digestion for 129 days, this serum contained but 2.5 per cent of its nitrogen in coagulable form, while the biuret action is feeble, indicating that nearly all the protein has been decomposed into free amino-acids except a small portion which obstinately remains as a soluble protein, apparently possessed of a great resistance to trypsin. A sample of this material called "Digestion Mixture IV" gave the following results:

TABLE 14.

	First Injection	Interval	Second Injection	Results
1...	1.0 c.c. Digest. Mix. IV	25 days	4 c.c. bovine serum	Death in 45 min.
2...	0.1 " " "	25 "	4 " "	Death in 45 min.
3...	0.02 " " "	25 "	4 " "	Doubtful symptoms
4...	0.01 " " "	25 "	4 " "	No symptoms
5...	0.005 " " "	25 "	4 " "	No symptoms
6...	0.2 " " "	26 "	5 dog serum	No symptoms
7...	1.0 " " "	26 "	0.2 gm. egg albumin	No symptoms
8...	0.5 " " "	21 "	5.0 c.c. Digestion Mixture IV	No symptoms
9...	0.02 bovine serum	21 "	5 " "	No symptoms
10...	0.02 " " "	21 "	5 " "	No symptoms
11...	0.02 " " "	80 "	1 Digest. Mixt. IV (intracar.)	No symptoms
12...	0.02 " " "	40 "	1 " " " "	Death in 4 min.
13...	0.5 Digest. Mix. IV	21 "	1 " " " " (intracar.)	Death in 2 min.
14...	0.5 " " "	21 "	1 " " " " "	Death in 3 min.
15...	0.5 " " "	21 "	1 " " " " "	Death in 4 min.
16...	0.1 gm. egg albumin	60 "	1 " " " " "	No symptoms

Evidently between the 59th and 129th days of digestion the only change has been a slight reduction in the sensitizing power so that

$\frac{1}{50}$ c.c. scarcely produces effects, while $\frac{1}{10}$ c.c. sensitizes fatally. This latter preparation also fails to sensitize to heterologous proteins (dog serum and egg albumin), and these heterologous proteins do not render the animals refractory to a dose of bovine serum given 24 hours later. The digestion of this serum is being continued and the eventual changes will be studied and reported. Similar studies with pure crystallized egg albumin are also under way.

MISCELLANEOUS OBSERVATIONS.

A few miscellaneous observations have been made in the course of these experiments, which, although not bearing directly upon the problems in hand, should probably be included in this report.

One concerns the mutual sensitizing and intoxicating power of bovine serum and bovine milk. As both serum and milk in this case come from the same species, it is of interest to consider their relation to one another in respect to anaphylaxis. The following experiments seem to indicate that this relation is a very uncertain one, the one fatality standing alone.

TABLE 15.

	First Injection	Interval	Second Injection	Results
1.....	0.02 c.c. bovine serum	40 days	5 c.c. milk	Slight symptoms
2.....	0.02 " "	57 "	10 "	Died in 110 min.
3.....	0.02 " "	46 "	10 "	No symptoms
4.....	0.02 " "	40 "	10 "	No symptoms
5.....	0.02 " "	46 "	10 "	No symptoms
6.....	1 milk	19 "	5 bovine serum	Slight symptoms
7.....	1 "	19 "	5 " "	Slight symptoms

Animals recovering from milk after bovine sensitization (Nos. 1, 3, 4, and 5) were found to be fully sensitive to bovine serum when this was injected 24 hours later, showing that the milk had not rendered them refractory to serum.

The second is an observation upon the effect of preservation upon horse serum. Rosenau and Anderson state that preservation of horse serum for $8\frac{1}{2}$ years does not appreciably alter its intoxicating effect. A specimen of horse serum that had been preserved for about fifteen months in an ordinary refrigerator, with the use of chloroform in the serum, was kindly furnished me by Dr. N. MacL. Harris, and in the course of certain studies the following observations were made:

TABLE 16.

	First Injection	Interval	Second Injection	Results
1...	0.01 c.c. old horse serum	18 days	5 c.c. bovine serum	Death in 2 hours, with typical symptoms
2...	0.1 " " "	18 "	5 " "	Death after 6 hours, following typical symptoms
3...	1.0 " " "	22 "	2 egg white	Moderate but typical symptoms. 24 hrs. later given 6 c.c. bovine serum and showed slight symptoms
4...	0.05 " " "	36 "	5 bovine serum	Moderate symptoms
5...	0.05 " " "	36 "	5 " "	Very slight symptoms
6...	0.05 " " "	36 "	5 dog serum	Slight symptoms
7...	0.05 " " "	36 "	1.5 egg-white	Very severe symptoms
8...	0.05 " " "	36 "	1.5 " "	Very slight symptoms
9...	0.05 " " "	36 "	10 milk	No symptoms
10...	0.05 " " "	53 "	5 bovine serum	Moderate symptoms

All these pigs that recovered were given later 3 c.c. of horse serum each, which caused in each case more or less severe symptoms but none of the animals died. These experiments suggest that long preservation of serum under chloroform lessens somewhat its specificity as a sensitizing agent, for the degree of intoxication observed in several of these pigs was much more marked than is usual in experiments with heterologous proteins, and they are cited for what they are worth. As they do not immediately concern the work in hand, the suggestion that they offer has not been followed up. It may be mentioned that Obermayer and Pick noted that long standing under toluol alters serum in such a way that it produces precipitins that are at least quantitatively specific for the same toluol-altered serum, but the species specificity it preserved. In the above experiments the species specificity seems to be somewhat impaired, whether by the long action of the chloroform used in preservation, or by slow auto-digestion, or some other change, is not apparent.

SUMMARY.

Egg albumin, freed from the other proteins of egg-white by repeated crystallization, produces typically the anaphylaxis reaction. It sensitizes in doses as small as one-twenty-millionth of a gram; fatally in doses of one-millionth of a gram. The minimum lethal dose for sensitized pigs is about one-half a milligram by intraperitoneal injection, and about one-tenth to one-twentieth of a milligram when injected into the circulation.

The unpurified mixed proteins of egg-white are much less active than the pure albumin, the minimum sensitizing dose being about

one hundred times greater and the minimum lethal dose being five times greater than with purified egg albumin. This suggests the possible presence of inhibiting substances in crude egg-white.

So small is the quantity of egg albumin that is fatal to sensitized pigs, one-tenth of a milligram, or less, that it seems improbable that the injected protein itself can cause death by splitting up and liberating poisonous substances in the space of time in which this reaction occurs. More probably if poisonous substances are produced, they must be produced by the cells of the intoxicated animal from some of the substances in its own body, or the whole reaction may be essentially different from any ordinary intoxication.

The minuteness of the minimum sensitizing and intoxicating doses of pure egg albumin seems to indicate conclusively that, at least in the case of this particular protein, both the sensitizing and the intoxicating agent are one and the same kind of protein molecule or else two different portions of the same molecule.

Gelatin seems to be devoid of the power of participating in the anaphylaxis reaction, either with itself or with other proteins. This may be due to its poverty in aromatic radicals; it probably is not due to the heating that is necessary for the conversion of collagen into gelatin. Addition of tyrosin to gelatin (without chemical combination) does not modify gelatin in respect to the anaphylaxis reaction.

Milk does not lose its sensitizing or intoxicating power when heated to 100° for 30 minutes. If large enough doses of serum heated to the same degree are used they will sensitize guinea-pigs to serum.

Coagulation with alcohol destroys or reduces greatly the toxicity of proteins which it renders insoluble in water (egg albumin) but not of proteins which it does not render insoluble (serum albumin).

Crystallized egg albumin does not entirely lose its sensitizing power when heated in aqueous solution to 100° for 15 minutes, if large doses are used. Heating to 90°, however, nearly destroys its intoxicating effect. Increasing the coarseness of the coagulated particles by addition of acetic acid seems to somewhat increase the effect of heating. Probably the destruction or reduction of toxicity of proteins by heat is due to the change in solubility of the proteins.

Pure zein is actively and specifically toxic to guinea-pigs sensitized with zein, although this protein is devoid of tryptophane and lysine.

Gliadin, which contains a less quantity of aromatic radicals than almost any other protein except gelatin, has but slight power to intoxicate pigs previously injected with gliadin.

Iodization of different specimens of serum by a constant method did not yield constant results. The partially saturated serum proteins did not suffer any modification in specificity analogous to that found by Obermayer and Pick in the case of the precipitin reaction. When most nearly saturated they may lose the power of sensitizing for the unaltered serum, but this is uncertain.

Pure crystallized egg albumin may be saturated with iodine quite readily, the iodine saturating the unsaturated carbon atoms of the benzene ring. Such iodized albumin retains its specificity unaltered, but seems to lose much of its toxicity for sensitized guinea-pigs, and its power to sensitize to egg albumin is impaired.

Tryptic digestion of serum furnishes further evidence of the protein nature of the substances concerned in the anaphylaxis reaction. Both sensitizing and intoxicating principles are attacked, and slowly decrease in strength as the coagulable protein disappears. After 59 days digestion of a sample of serum, so that but 4.7 per cent of the nitrogen was still in a coagulable form, the sensitizing dose had been changed from one-thousandth of a cubic centimeter to one-fiftieth of a cubic centimeter, while 5 c.c. intraperitoneally did not intoxicate pigs previously sensitized to bovine serum. Digestion of serum to this extent does not affect its specificity for species, but the digested serum sensitizes much better to itself than to undigested bovine serum, and conversely, it intoxicates pigs sensitized to itself much more than it intoxicates pigs sensitized to unaltered bovine serum.

Possibly the specificity of horse serum suffers somewhat after prolonged preservation with chloroform.